Electronic Supplementary Information (ESI)

Hyaluronic Acid Grafted Nanoparticles of a Platinum(II)-Silicon(IV) Phthalocyanine Conjugate For Tumor and Mitochondria Targeted Photodynamic Therapy in Red Light

Koushambi Mitra,[a,b] Montserrat Samso [c], Charles E. Lyons,[b] and Matthew C. T. Hartman*[a,b]

a. Department of Chemistry, Virginia Commonwealth University

b. Massey Cancer Center, Virginia Commonwealth University

c. Department of Physiology and Biophysics, Virginia Commonwealth University

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1. Materials and methods

All chemicals (solvents and reagents) were purchased from commercial sources (Sigma Aldrich, Fisher Scientific and VWR) and used without further purification. Solvents were dried using standard protocols. Reactions were carried out in argon atmosphere and in Al foil-wrapped light-protected conditions, unless otherwise stated. NMR spectra were recorded using a 400 MHz Bruker Avance Varian spectrometer. Electrospray ionization mass spectroscopy (ESI-MS) was performed with "Thermo Finnigan LCQ deca XP max" instrument. High resolution mass spectroscopy (HRMS) analysis was done using the HESI source of the Orbitrap Velos mass spectrometer purchased from Thermo Electron Corporation. The dynamic light scattering measurements (DLS) were carried out using the Zetasizer Nano Series (Nano-ZS, Malvern Instruments Ltd., U.K.). The DLS light source used was a He-Ne laser (633 nm, max 4 mW). TEM images were captured using a Thermo Fisher Tecnai F20 electron microscope. Absorption and fluorescence measurements were done using Varian Cary Eclipse and Agilent spectrophotometers. A Varian ICP 820-MS instrument was utilized to determine the platinum content by inductively coupled plasma mass spectrometric (ICP-MS) method. MTT readings were taken with a BioTek Synergy H1 hybrid 96-well plate reader. Fluorescence activated cell sorting (FACS) data was obtained by running samples on Canto - BD FACSCanto™ II Analyzer instrument, equipped with BD FACS carousel loader and 2 lasers (blue: 488 nm, red: 633 nm). Post-experiment data analysis was performed with FACSDIVA and FCSExpress 5 flow softwares. Confocal microscopic images were captured using LSM710 Zeiss instrument (63X magnification using oil-immersion objective) and processed with Zen and ImageJ softwares. The photo-exposed experiments were carried out using commercially available LEDs (660-680 nm, RapidLED, Solderless Cree XP-E Red LED)

with an average output of (5.5±2.5) $mW.cm^{-2}$. The intensity of the LED source was measured using a Thorlabs PM100 optical power and energy meter.

2. Experimental Procedures

Synthesis:

SiPc-1. The silicon phthalocyanine dichloride, $SiPcCl_2$ (200 mg, 0.33 mmol) was refluxed in dry toluene (15 ml) with excess of nicotinic acid (200 mg, 1.6 mmol) for 24 h in inert and light protected conditions. The solution was cooled down and filtered. The precipitate was extracted with chloroform (5 x 100 ml) and evaporated to obtain a deep blue solid. It was washed with acetonitrile and dried to obtain the desired product in pure form. Yield = 40 mg, 20%. Molecular weight = 784.83 $g.mol^{-1}$. ^1H NMR ($CDCl_3$): δ = 9.65 (m, 8H); 8.34 (m, 8H); 7.70 (t, 2H, $J_1 = J_2 =$ 4 Hz); 6.16 (m, 2H); 5.95 (s, 2H); 5.66 (m, 2H). HRMS: Expected m/z for [M+H]+ = 785.1830; observed m/z for $[M+H]^+$ = 785.1774. The ^{13}C NMR spectra could not be obtained even after long overnight scans (ns ~ 15,000) due to poor solubility in deuterated chloroform.

SiPc-Pt. Cisplatin (200 mg, 0.67 mmol) was reacted with one equivalent of silver nitrate (110 mg, 0.70 mmol) in dry DMF (10 ml) and stirred at room temperature for 20 h in argon atmosphere. The resulting curdy white precipitate was filtered off. To the pale yellowish filtrate, **SiPc-1** (110 mg, 0.18 mmol) dissolved in dry DMF (10 ml) was added dropwise. The reaction was stirred at 50 °C for 12 h, protected from light. The solution was cooled to room temperature and filtered. Cold diethyl ether (200 ml) was added to the blue filtrate and allowed to stand for 3 h in an ice-bath. The resulting precipitate was collected and washed sequentially with water (5 ml), acetone (5 ml) and diethyl ether (10 ml) and dried to obtain the desired product as a bright blue precipitate. Yield = 100 mg, 30%. Molecular weight = 1438.03 $g.mol^{-1}$. ^1H NMR (DMSO-d_6): δ = 9.75 (m,

8H); 8.59 (m, 8H); 7.98 (d, 2H, *J* = 4.8 Hz); 6.81 (s, 2H); 6.49 (m, 2H); 5.16 (d, 2H, *J* = 8.4 Hz), 3.91 (s, 6H); 3.88 (s, 6H). ^{13}C NMR (DMSO-d_6): δ = 155.0, 151.9, 136.9, 135.1, 135.0, 133.1, 127.5, 125.3, 124.6. HRMS: Expected m/z for $[M\text{-}2(NO_3)]^{2+}$ = 656.0743; observed m/z = 656.0665.

SiPc-Pt-HA. **SiPc-Pt** (5 mg) dissolved in DMF (0.5 ml) was added in a dropwise manner to a solution of HA (10 mg) in double distilled water (15 ml) with continuous stirring. After 15 min, double distilled water (5 mL) was added to the solution and was stirred for 12 h at room temperature. The solution was centrifuged, and the supernatant was collected and dialyzed using a 3.5KD cut-off membrane tube in double distilled water for 24 h to obtain the **SiPc-Pt-HA** nanoparticles.

DLS measurements: The hydrodynamic size and size distribution in terms of the polydispersity index (PDI) of particles (sizes ranging from 1 nm to 5 μm) in solution can be evaluated using dynamic light scattering (DLS). The basic principle of DLS involves probing a colloidal suspension with a monochromatic light source and then recording the time variation of the intensity of the light scattering by the nanoparticles that are diffusing in a solution. The intensity autocorrelation function is then used to express this data in terms of the correlation between the intensity measured at one time point and the intensity after a delay in time. The diffusion coefficients are used to calculate the hydrodynamic diameter and PDI values. For DLS analysis, solutions of **SiPc-Pt-HA** were transferred to a low volume disposable cuvette and the temperature was allowed to be equilibrated at 25 °C for 60 s before each measurement. The hydrodynamic diameter and PDI values were determined using standard operating procedure, as an average of 3 runs, each individual run having 12 measurements. The collected data were analyzed using

Malvern Zetasizer Software with the General Analysis Mode. The hydrodynamic diameters and the PDI values were directly obtained automatically from analysis by the in-built software.

TEM images: For transmission electron microscopy, sample was prepared on continuous carbon films supported on 400 mesh copper grids (Ted Pella, Redding, CA). A 3 μL drop of sample solution was applied to a freshly plasma-cleaned grid for 40 s and blotted to a thin film using filter paper and finally air-dried. Sample was imaged on a Thermo Fisher Tecnai F20 electron microscope operated at 120 kV under low-dose conditions (electron doses below 20 e/Å^2) and images were recorded on a Gatan 4k x 4k charge-coupled-device (CCD) camera. Images were acquired at underfoci ranging between 0.5-2.5 μm.

Singlet oxygen quantum yields: The singlet oxygen quantum yields were determined by the 1,3-Diphenyl isobenzofuran (DPBF) quenching assay in aerated DMF solutions.[S1] Light exposure was performed with 660-680 nm LEDs for different time intervals depending on the rate of quenching of absorbance at 415 nm. The relative quantum yields of singlet oxygen generation (φ_Δ) were calculated by using the equation: $\varphi_\Delta = \varphi_\Delta^{ref}$ (k/ k^{ref})(I_a^{ref}/I_a), in which φ_Δ^{ref} is the singlet oxygen quantum yield for the standard (zinc phthalocyanine, φ_Δ =0.56), k and k_{ref} are the photobleaching rate constants of DPBF, and I_a and I_a^{ref} are the absorption intensities (690 nm) of the compounds.

Pt estimation by ICP-MS methods: Calf-thymus DNA, ctDNA (500 μM) was treated with **SiPc-Pt** or cisplatin (50 μM in 10% DMSO-PBS) and either exposed to red light (660-680 nm) for 4 h or kept in dark for 24 h. The Pt-bound DNA was isolated as a white fibrous material using precipitation method by cold ethanol. The precipitate was digested in concentrated HNO_3 (200 μL) for 12 h and then distilled water was added to make a 2% HNO_3-water solution. These samples along with untreated ones (DNA alone, complex alone) were measured for Pt content by ICP-MS.

Standard samples of known Pt concentrations were used for calibration. The data along with the deviation is reported based on experiments performed in duplicate.

Cellular Uptake: MDA-MB-231 and HEK293T cells (~ 10^6) were plated in six well plates and allowed to attach for 24 h at 37 °C and 5% CO_2. Cells were then treated with compounds, **SiPc-Pt** or **SiPc-Pt-HA** (10 μM in 0.5% DMSO-DMEM) for 4 h, after which the media was aspirated. Cell were washed with DPBS, trypsinized and collected by centrifugation. The pellets were dissolved in 500 μL 1X PBS and homogenized to form single cell suspensions. Flow cytometric analysis of these samples was measured using excitation and emission channels in the red region. Identical experiments were performed with pre-treatment of the cells with hyaluronic acid (500 μM, 2 h) to identify the mechanistic pathway of cellular uptake. All the experiments were performed in duplicate.

Confocal microscopy: Confocal microscopic images (63X) were recorded using oil immersion lens of LSM710 Zeiss instrument. MDA-MB-231 and HEK293T cells (~ 10^5) were allowed to adhere to cover slips in a 12-well tissue-culture plate for 24 h. They were treated with compounds, **SiPc-Pt** or **SiPc-Pt-HA** (10 μM in 0.5% DMSO) for 4 h in light-protected conditions. After treatment, the media was aspirated, cells were washed with PBS and stained first with Mito-Tracker® Green (50 nM in PBS) for 15 mins at room temperature. Cells were washed and then stained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, nuclear stain, 300 nM in PBS) for 5 min at room temperature. Cells were thoroughly washed to remove the excess dyes. These dye-stained cover slips were transferred carefully to slides containing AntifadeGold reagent (1 μl). They were fixed by coating the sides with quick-dry transparent nail-enamel. Cells treated with only 0.5% of DMSO were also stained with the dyes and used as controls to eliminate cellular

auto-fluorescence and background signals. Experiments were performed in duplicate and multiple images were recorded from each cover slip to confirm the results.

MTT assay: The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a colourimetric assay widely used to determine cell viability.[S2] Cytotoxic profiles of the compounds were assessed in the following cell lines (passage number not exceeding 15): MDA-MB-231 (human triple-negative breast cancer) and HEK293T (transformed human kidney). Approximately 8000 cells were seeded in 96 well tissue-culture plate in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and were incubated for 24 h at 37 °C and 5% CO_2. Cells were treated with various concentrations of **SiPc-Pt-HA**, **SiPc-Pt**, **SiPc-1** and cisplatin in 0.5% DMSO-DMEM for 4 h in absence of light. One such plate was exposed to red light (660-680 nm, 5.5 ± 2.5 $mW.cm^{-2}$) for 1 h in phenol-red free DMEM while another identical plate was kept unexposed in similar conditions. The plates were incubated for another 24 h in dark, after which MTT (25 μl of 5 mg/ml in PBS) was added and further incubated for 4 h at 37 °C. The violet precipitate was dissolved in DMSO (200 μl) and the absorbance was recorded at 550 nm. Identical experiments were performed with cells incubated with the compounds for longer period of 24, 48 or 96 h without exposure to red light. Cells treated with 0.5 % DMSO-DMEM did not show any significant cell death as compared to cells alone. The inhibitory concentration (IC_{50}) values were determined by nonlinear regression analysis using GraphPad Prism5 and obtained from three independent set of experiments, each of which was performed in triplicate for each concentration.

DCFDA assay: DCF (2,7-dichlorofluorescein) is formed in cells as a result of oxidation of the diacetate analogue, DCFDA by reactive oxygen species (ROS). DCF is emissive at 525 nm, while the DCFDA is not fluorescent. Thus, amount of ROS generated in a cell can be quantified by measuring fluorescence intensity at 525 nm cells treated with DCFDA.[S3]

MDA-MB-231 cells with a seeding density of ~ 10^6 were plated in 6-well plates and allowed to grow for 24 h. Cells were then incubated with **SiPc-Pt-HA** (1 μM in 0.5% DMSO-DMEM) for 4 h in light protected conditions. After treatment, the media was replaced with phenol-red free DMEM and cells were irradiated (660-680 nm, 45 mins). An identical counterpart was kept unexposed. Cells were then trypsinized, collected and re-suspended in PBS. DCFDA (1 μM) was added to the cells and kept for 20 min at room temperature. DCFDA stained and unstained cells treated only with 0.5% DMSO were kept as control. All samples were subjected to flow cytometry to obtain the data represented from experiments done in duplicate.

Apoptosis assay: The annexinV-FITC (fluorescein isothiocyanate)/ PI (propidium iodide) assay is based on preferential uptake of the dyes by live, early apoptotic, late apoptotic and dead cells. The early apoptotic cells are permeable only to annexinV-FITC dye, while the late apoptotic/ necrotic cells are permeable to both annexinV-FITC and PI dyes.[S4]

Approximately 10^6 MDA-MB-231 cells were plated in a six well plate and allowed to attain ~70% confluency. Cell were then incubated with **SiPc-Pt-HA** (1 μM in 0.5% DMSO-DMEM) for 4 h in light protected conditions. After treatment, the plate was photo-exposed to red light for 45 mins in phenol-red free media. An identically treated plate was kept in dark. Media was then replaced with 10%-DMEM-FBS and cells were incubated for another 12 h in dark. Post-processing was carried out by trypsin treatment and subsequent centrifugation. Cells were re-suspended in 500 μL of 1X binding buffer and were stained using AnnexinV-FITC (1 μL) and PI (0.5 μL) using protocols from the AnnexinV-FITC apoptosis detection kit (Sigma Aldrich, APOAF-20T ST). The green and red emission intensities of these samples were determined with flow cytometry. Cells treated with 0.5% DMSO were either unstained or stained with dyes (PI alone, AnnexinV-FITC alone,

both PI and AnnexinV-FITC) were used for gating purpose. All experiments were performed in duplicate to confirm the results.

3. References:

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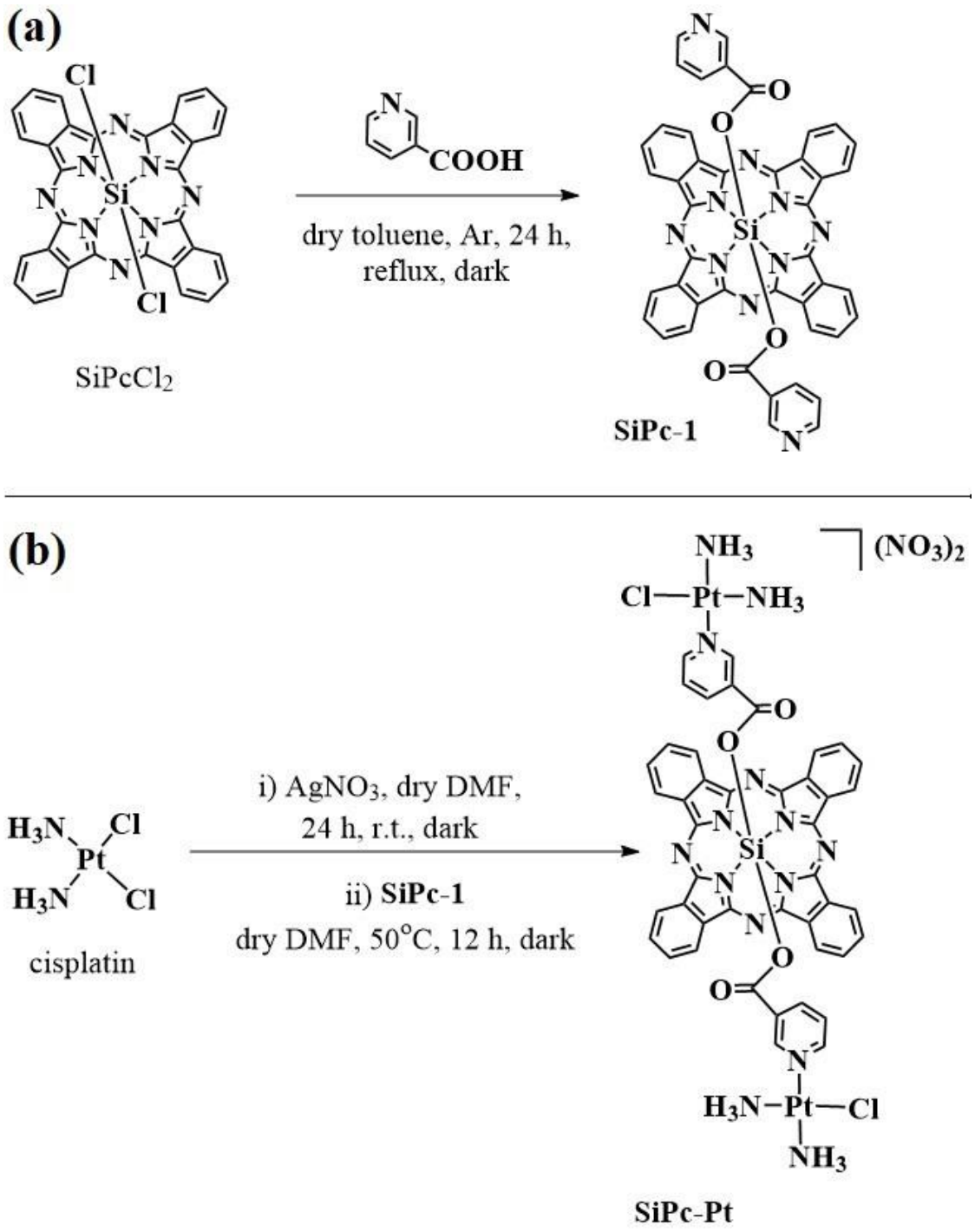
S2. T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55– 63.

S3. M. Price, D. Kessel, *J. Biomed. Opt.* **2010**, *15*, 0516051.

S4. I. Vermes, C. Haanen, H. Stefens-Nakken, C. Reutelingsperger, *J. Immunol. Methods* **1995**, *184*, 39– 51.



Scheme S1. Scheme showing synthesis of (a) **SiPc-1** and (b) complex **SiPc-Pt**.

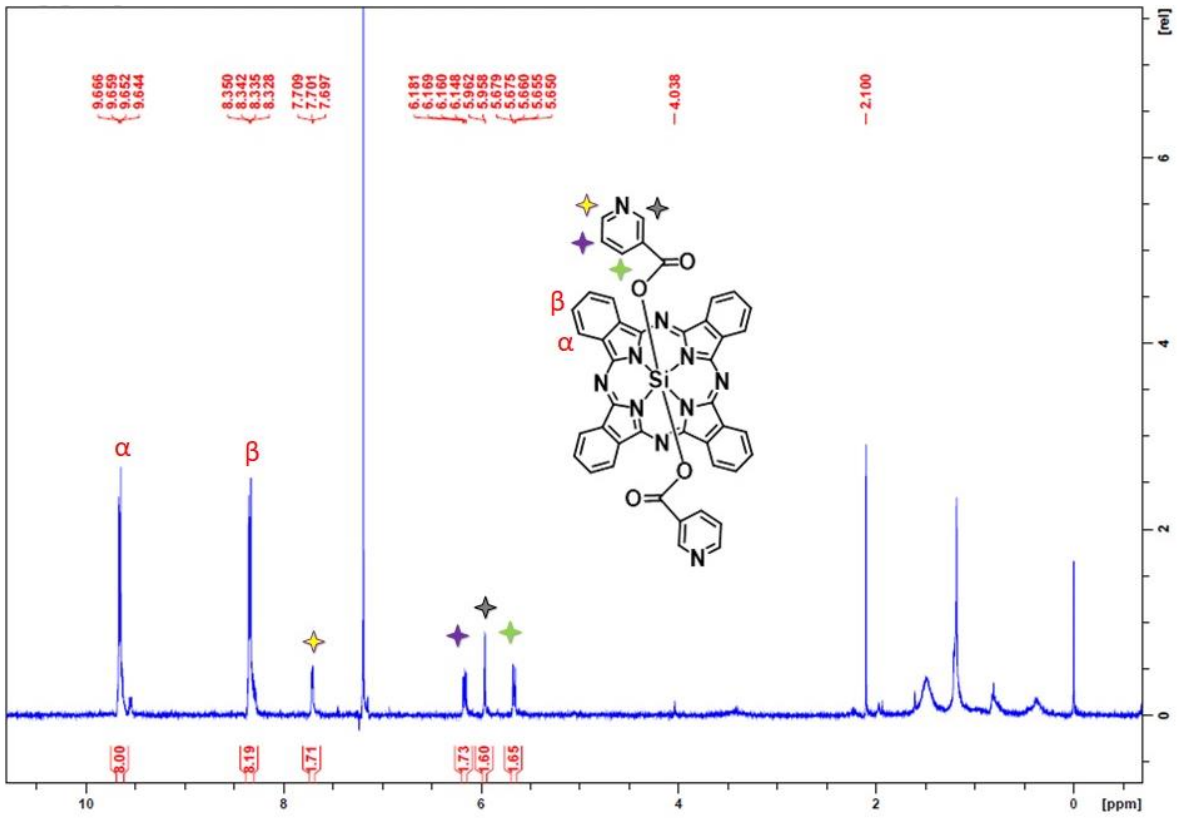


Fig. S1. ^{1}H NMR spectra of **SiPc-1** in $CDCl_3$.

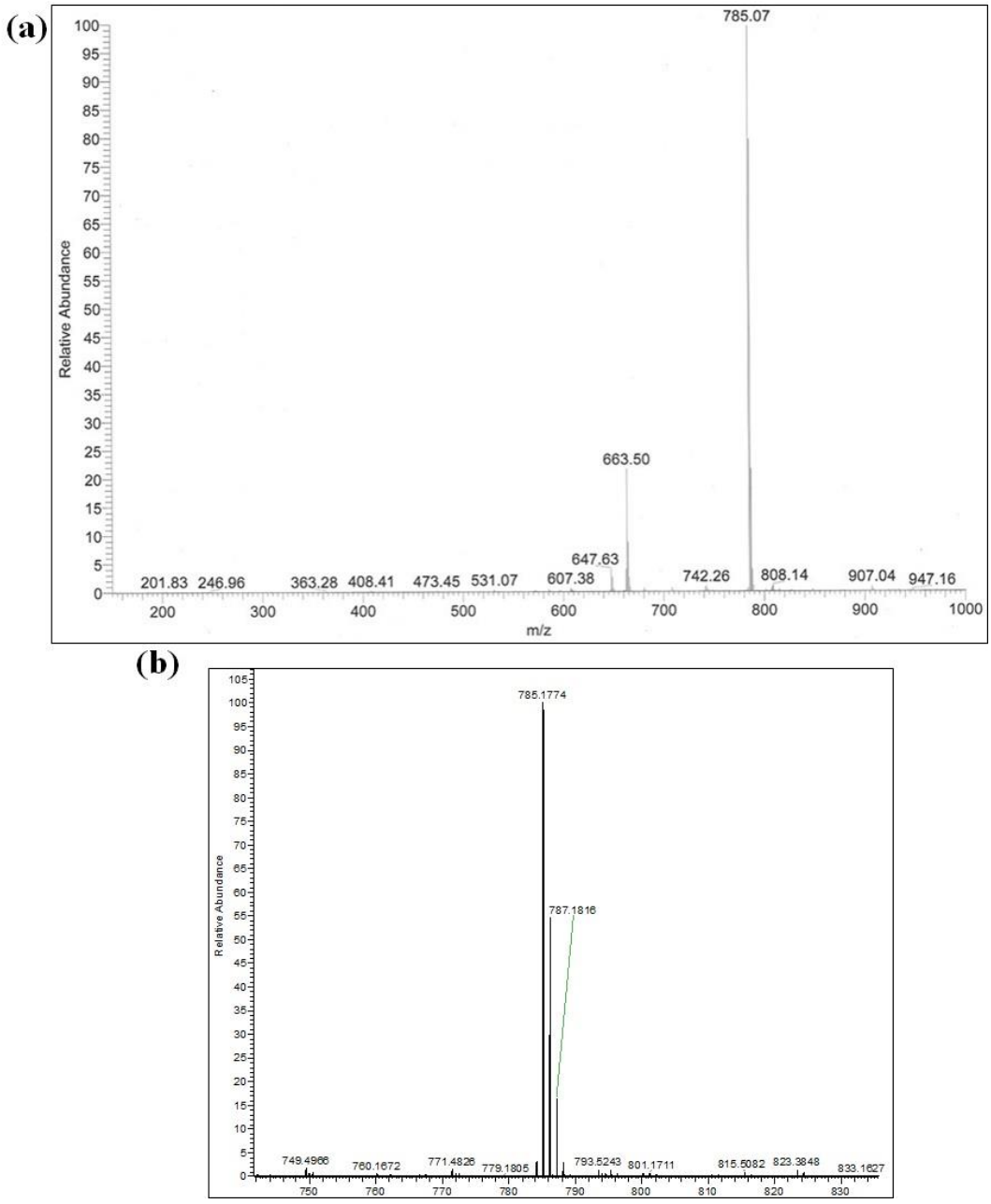


Fig. S2. (a) ESI-MS of **SiPc-1** in MeOH-$CHCl_3$ solvent mixture. The peak at m/z = 785.07 corresponds to $[M+H]^+$ species. The minor peak at 663.50 corresponds to a fragment assignable to the compound without one axial ligand. This fragmentation occurred under mass spectral conditions. (b) HRMS showing most abundant peak for **SiPc-1**.

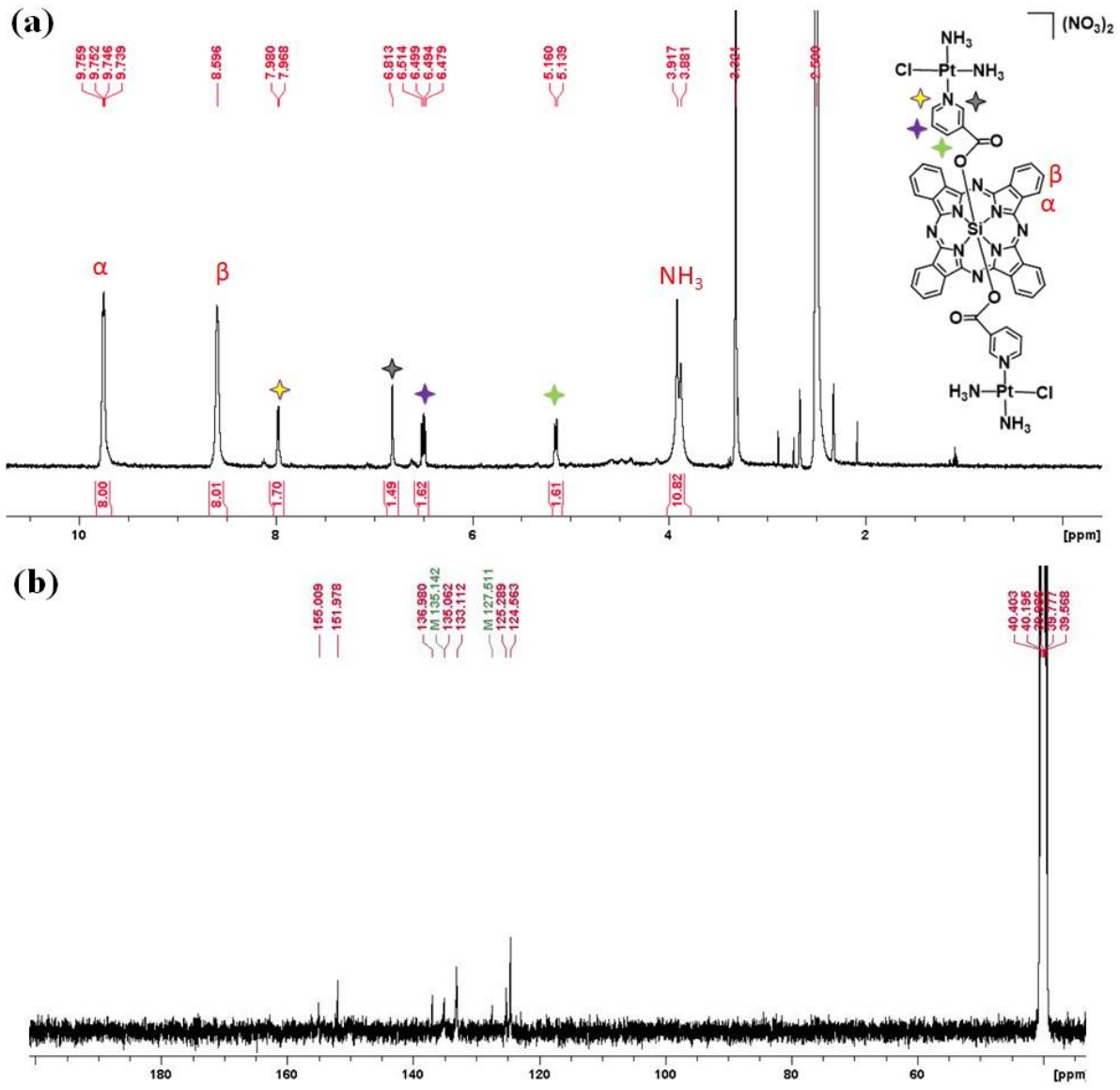


Fig. S3. (a) ^{1}H and (b) ^{13}C NMR spectra of **SiPc-Pt** in DMSO-d_6.

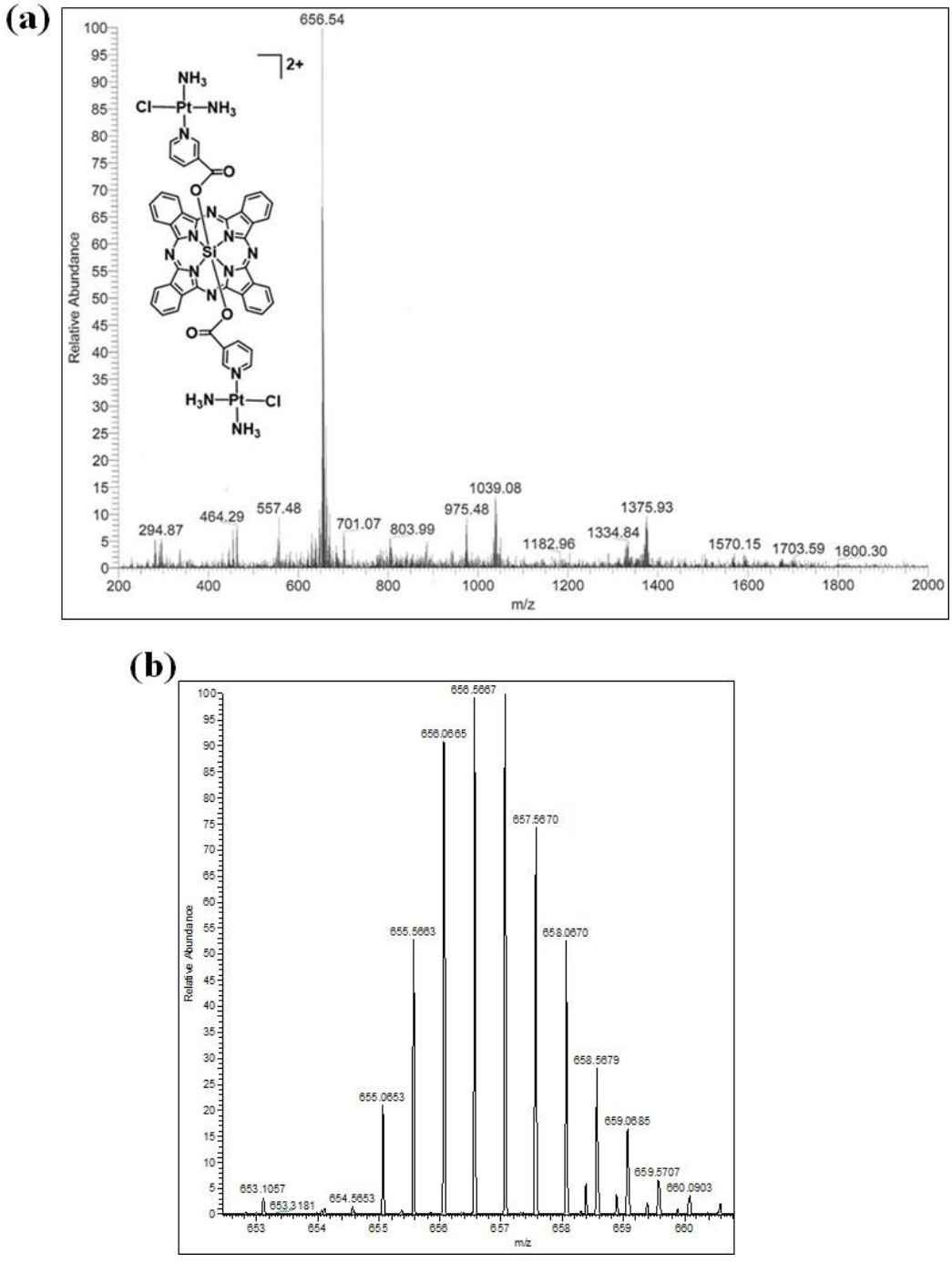


Fig. S4. (a) ESI-MS of **SiPc-Pt** in acetonitrile. The peak at m/z = 656.54 corresponds to $[M-2(NO_3)]^{2+}$ species. (b) HRMS of **SiPc-Pt** in acetonitrile showing the Pt isotopic pattern.

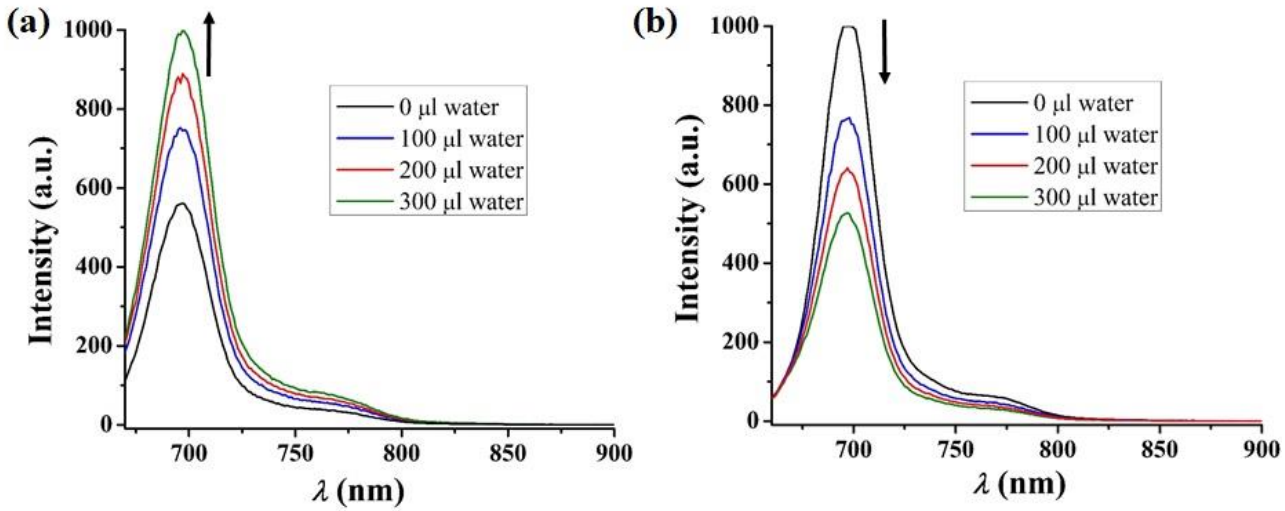


Fig. S5. Changes in emission intensities of (a) **SiPc-1** and (b) **SiPc-Pt** (10 µM in 3 ml of 20% DMF-PBS solution) on dilution with water. Readings were taken after serial dilutions by adding water (100 µl of water added in succession) as depicted in the graph. The excitation wavelength is 650 nm. **SiPc-1** showed increase in emission intensity while **SiPc-Pt** showed a gradual decrease on addition of water.

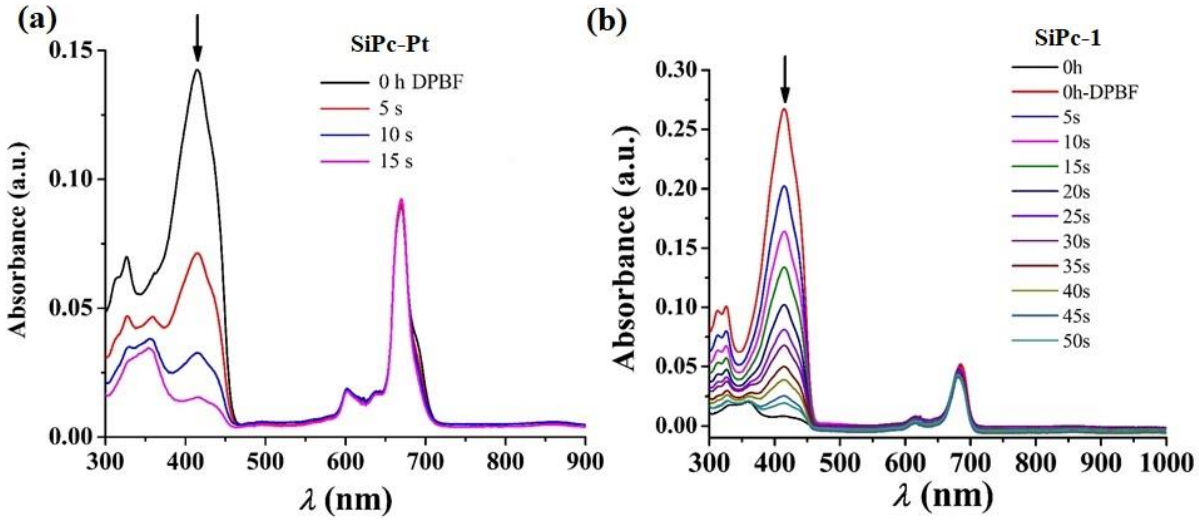


Fig. S6. Singlet oxygen generation by (a) **SiPc-Pt** and (b) **SiPc-1** in DMF as evident from the decrease in absorbance of DPBF (a singlet oxygen quencher) at 415 nm on red light irradiation (660-680 nm, 5.5 ± 2.5 mW cm^{-2}) at different intervals as depicted in the graph.

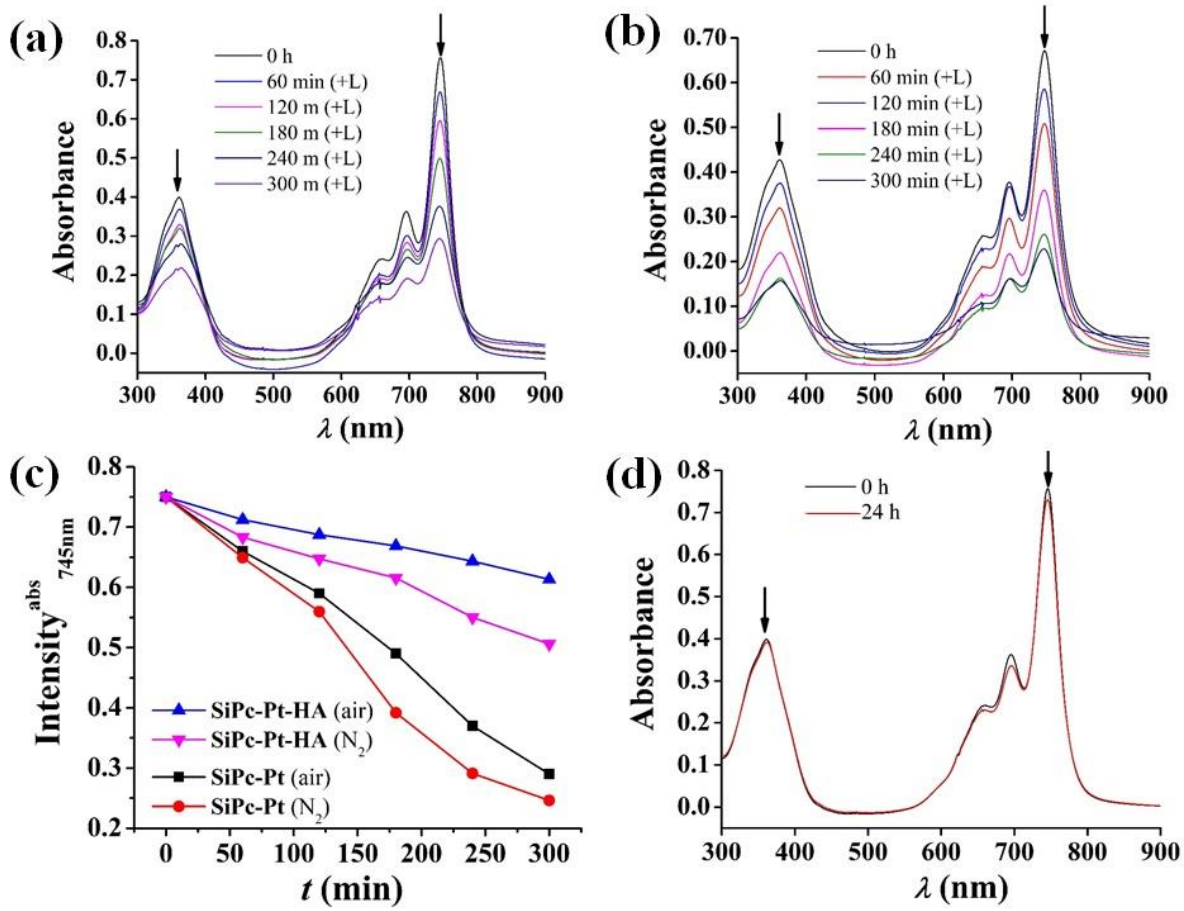


Fig. S7. UV-visible spectra of **SiPc-Pt** (10 μM in 10% DMF-PBS, pH, 7.4) in (a) air and in (b) nitrogen atmosphere demonstrating photo-instability when exposed to red light (+L, 660-680 nm). The complex was found to be stable in absence of light (-L) under similar conditions. (c) Plot showing decrease in normalized absorbance at 745 nm of **SiPc-Pt-HA** and **SiPc-Pt** solution when exposed to red light either in air or in nitrogen (N_2) atmosphere. (d) Stability of **SiPc-Pt** (10 μM in 10% DMF-PBS, pH, 7.4) in absence of light (-L) for 24 h.

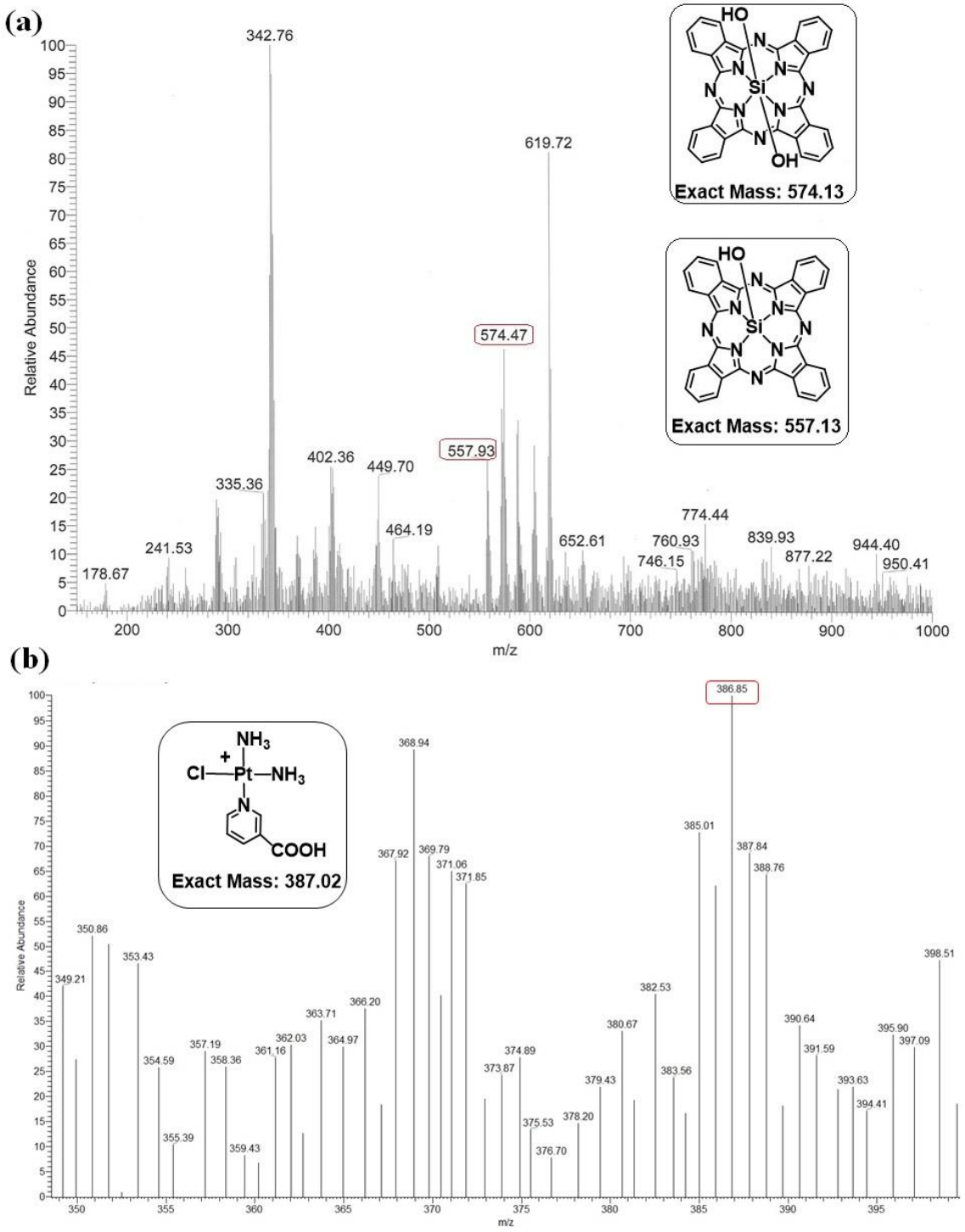


Fig. S8. ESI-MS of photolyzed samples of **SiPc-Pt** (3 h irradiation in red light of 660-680 nm in 10% DMF-PBS) having mass spectral peaks assignable to the shown structures confirming the breaking of Si-O bonds and photo-removal of the axial groups. (a) Full spectra in the m/z range of 100-1000 units. (b) The same spectra zoomed in the m/z range of 350-400 units.

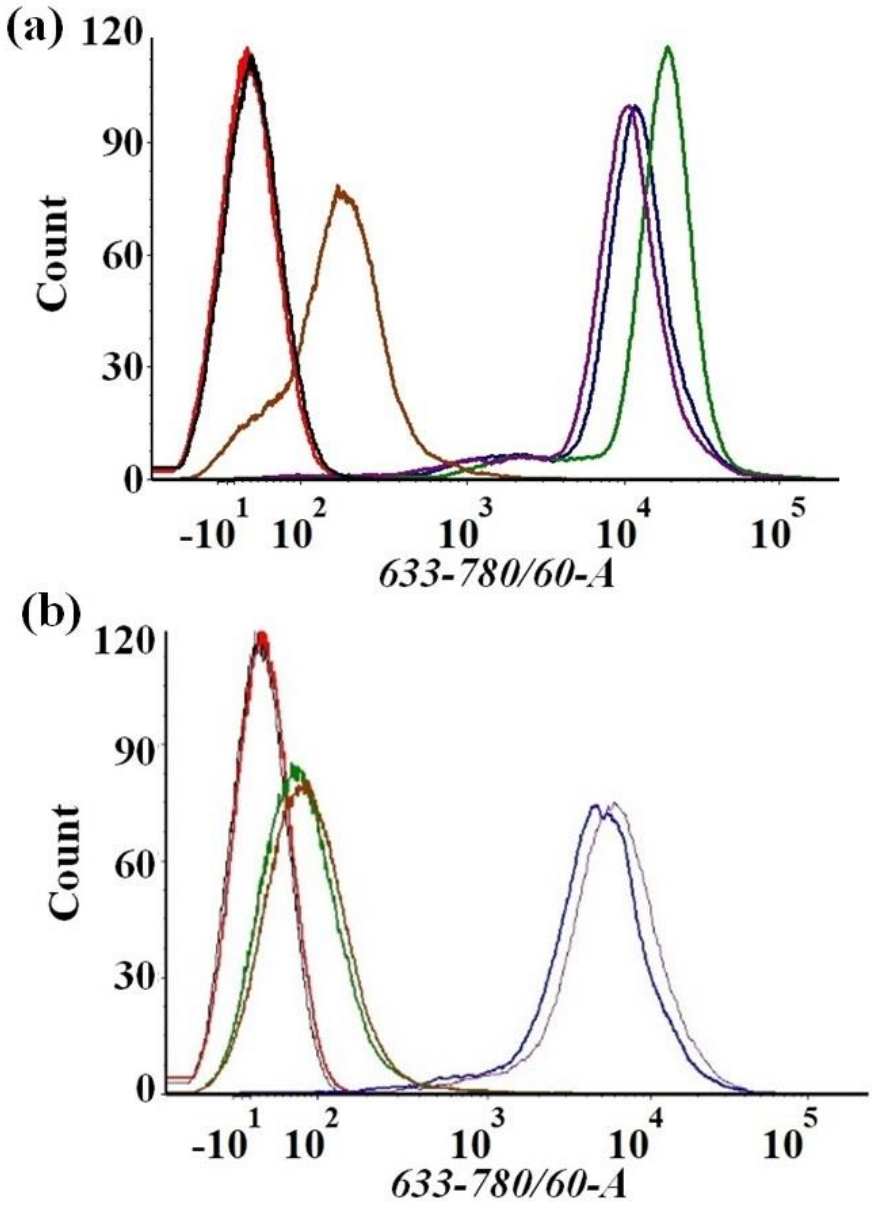


Fig. S9. Cellular uptake of **SiPc-Pt** and **SiPc-Pt-HA** in (a) MDA-MB-231 and (b) HEK293T cells (4 h) either as such or pretreated with hyaluronic acid (HA, 500 μM) as determined by measuring emission intensity in the red channel with flow cytometric techniques. Color codes: black, cells - HA; red, cells + HA; purple, cells - HA + **SiPc-Pt**; blue, cells + HA + **SiPc-Pt**; green, cells - HA + **SiPc-Pt-HA**; brown, cells + HA + **SiPc-Pt-HA**.

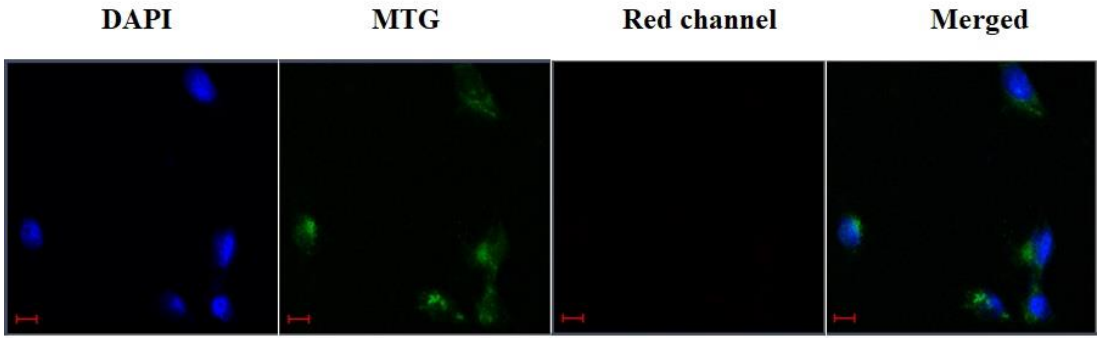


Fig. S10. Confocal microscopic images showing emission in untreated MDA-MB231 cells in the blue channel (DAPI, nuclear stain, first column), green channel (MTG, mitotracker green, second column), red channel (third column) and the overlap of all the three images (merged, fourth column). There was no emission detected in the red channel as expected for compound untreated cells. Scale bar = 10 μm.

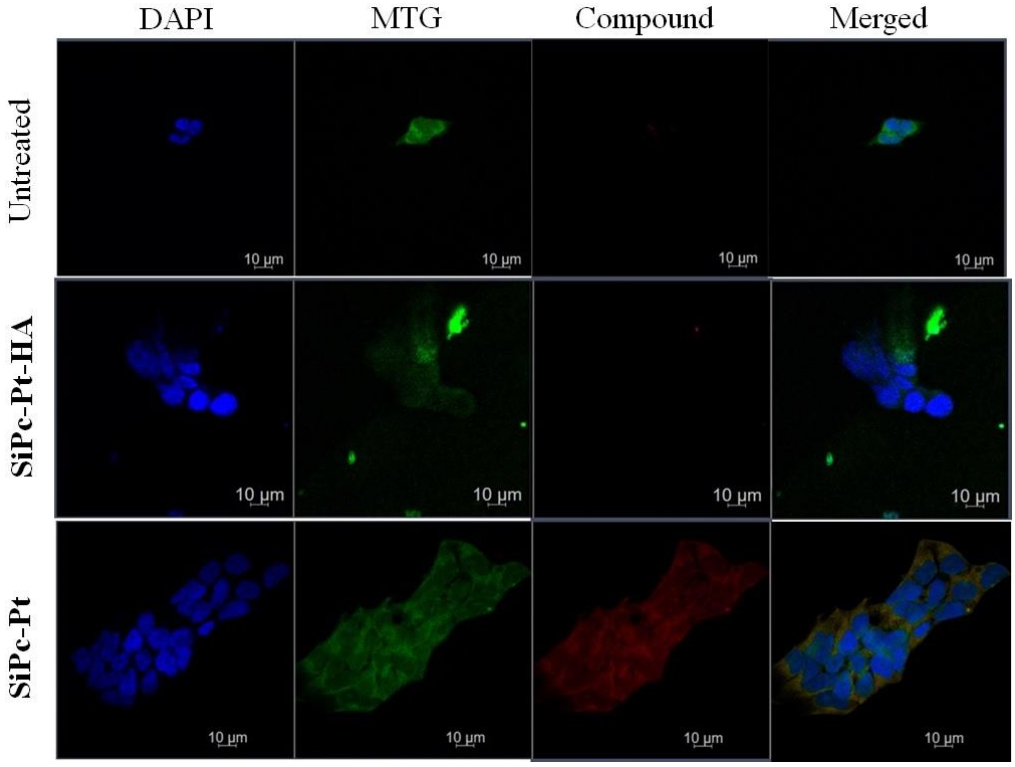


Fig. S11. Confocal microscopic images showing emission in HEK293T cells in the blue channel (DAPI, nuclear stain, first column), green channel (MTG, mitotracker green, second column), compound (red channel, third column) and the overlap of all the three images (merged, fourth column). There was no emission detected in the red channel as expected for untreated cells or cells treated with **SiPc-Pt-HA** nanoparticles (first row). The complex, **SiPc-Pt** showed mitochondrial accumulation (third row). Scale bar = 10 μm.

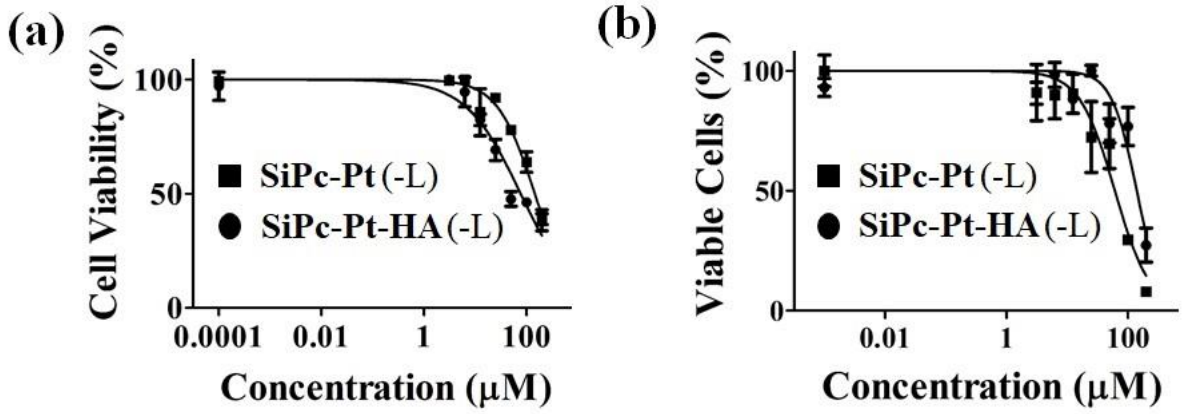


Fig. S12. Dose-dependent cellular viability (%) plots as obtained from MTT assay in (a) MDA-MB-231 and (b) HEK293T cells for **SiPc-Pt** and **SiPc-Pt-HA**. For evaluating the toxicities in absence of light irradiation (-L), cells were incubated with the compounds for either 96 h (MDA-MB-231) or 24 h (HEK293T) in the dark.

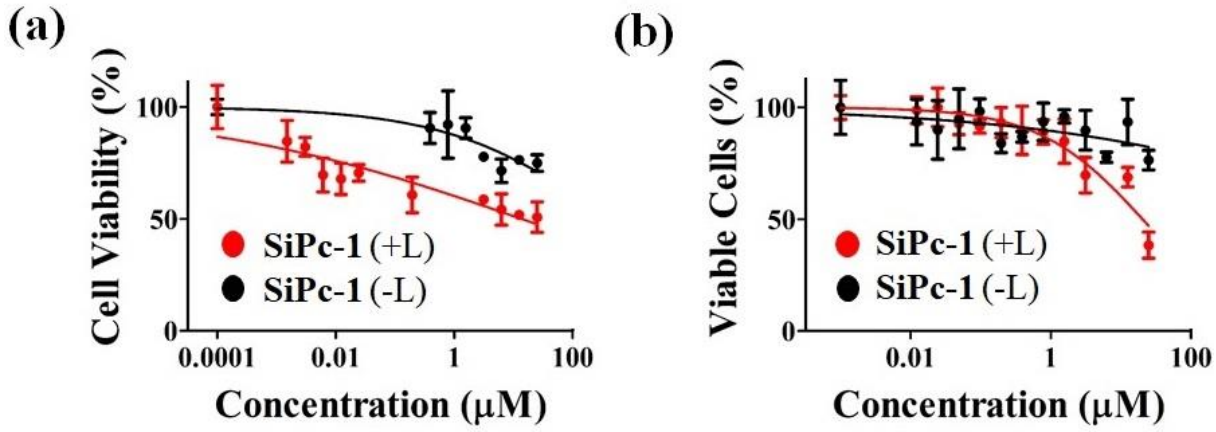


Fig. S13. Dose-dependent cellular viability (%) plots as obtained from MTT assay in (a) MDA-MB-231 and (b) HEK293T cells treated with **SiPc-1** for 4 h in dark. (+) L denotes experiments carried out with light exposure (red light, 660-680 nm, 45 mins) and (-) L is for experiments that were kept in light protected conditions.

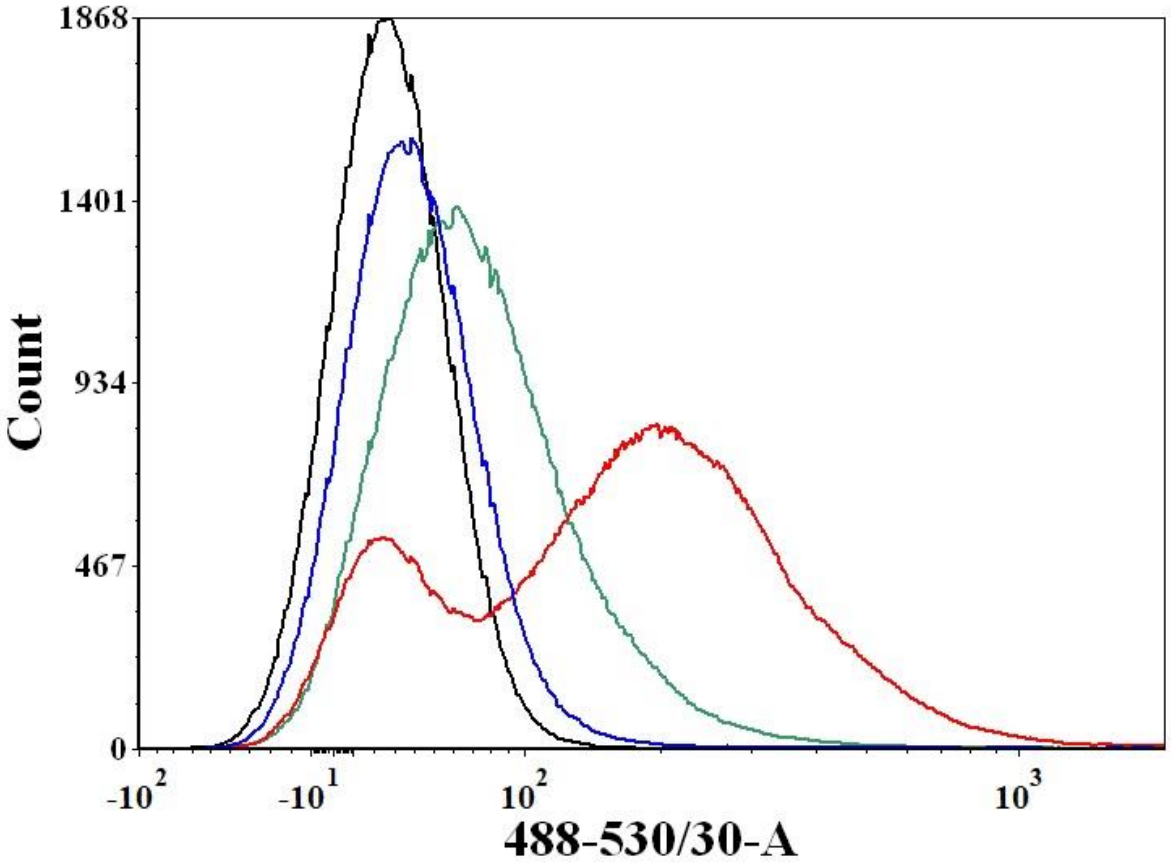


Fig. S14. DCFDA assay showing generation of reactive oxygen species (indicated by increase in emission intensity of DCF at 525 nm) in MDA-MB-231 cells treated with **SiPc-Pt-HA** (1 μM in 0.5% DMSO-PBS) for 4 h in the dark. Color codes: black, cells alone; blue, cells + DCFDA; cyan, cells + **SiPc-Pt-HA** (-light); red, cells + **SiPc-Pt-HA** (+light). The experiments (+light) were performed with photo-exposure using red light (660-680 nm, 45 mins), while (-light) was kept in light-unexposed conditions.

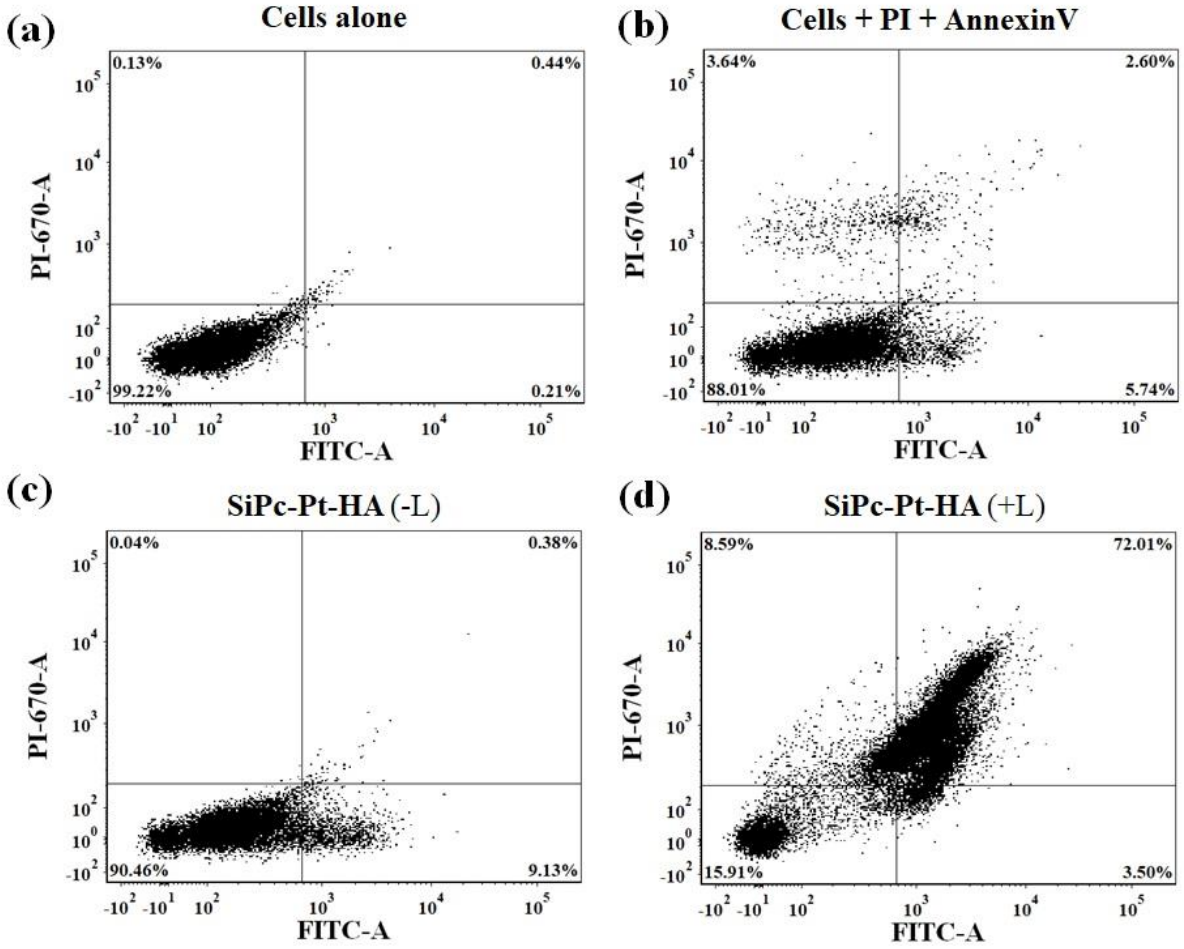


Fig. S15. Dot-plots as obtained from annexinV-FITC/ PI assay showing percentage population (depicted in the figures) of MDA-MB-231 cells treated with **SiPc-Pt-HA** (1 μM in 0.5% DMSO-PBS) for 4 h and either exposed to red light (+L, 660-680 nm, 45 mins) or kept in dark conditions (-L, 45 mins). The four quadrants denote the following: lower left, live cells; lower right, early apoptotic cells; upper right, late apoptotic/ necrotic cells; upper left, dead cells. Cells treated with **SiPc-Pt-HA** showed late apoptotic features when exposed to red light.

Table S1. Spectral properties of SiPc-Pt-HA, SiPc-Pt and SiPc-1

Compound	λ_{abs} (nm), [ε x 10^{-4} (M^{-1}. cm^{-1})][a]	λ_{em} (nm), [a] [φ_f] [b]	φ_Δ ($t_{1/2}^{DPBF}$/ sec) [c]
SiPc-Pt-HA	744 [11.8], 695 [8.6], 650 [6.2], 360 [10.1]	705 [n.d.]	0.24 (7)
SiPc-Pt	690 [11.6], 620 [2.5], 360 [5.8]	700 [0.20]	0.22 (10)
SiPc-1	675 [12.1], 640 [6.1], 345 [6.8]	690 [0.31]	0.15 (16)

[a] Absorption and emission spectra recorded in DMF or in double distilled water. [b] Fluorescence quantum yields measured in DMF using zinc phthalocyanine as standard (φ_f = 0.3). [c] Singlet oxygen generation and half-life of 1,3-diphenylisobenzofuran (DPBF) recorded in DMF using zinc phthalocyanine as reference (φ_Δ = 0.56). The singlet oxygen quantum yield value determined for **SiPc-Pt-HA** has high experimental error due to inherent light scattering phenomena associated with nanoparticles in solutions. n.d. = not determined.